

2. AUG. 2006 9:01

DOMPATENT VON KREISLER KOELN

NR. 2310 S. 3/61

Express Mail No. EV529815955US

**10/588570****IAP11 Rec'd PCT/PTO 03 AUG 2006**

SMB

**Mutated DNA Polymerases with Increased Mispairing Discrimination**

The present invention relates to DNA polymerases with a special mutation which have an enhanced mismatch discrimination, the preparation and use thereof. The thermostable DNA polymerases with this mutation are particularly suitable for diagnostic and molecular-biological methods, e.g., allele-specific PCR.

**Background of the Invention**

Since the first human genome sequences were presented, the research has been focused on the discovery of genetic differences between individuals, such as single-base mutations ("single nucleotide polymorphisms", SNPs). This is of interest because it becomes more and more evident that single-base variations in the genome are associated with different drug tolerances or predisposition for a wide variety of diseases. In the future, the knowledge of medically relevant nucleotide variations could allow to adapt therapies to the individual genetic supply, and treatment with medicaments which are ineffective or even cause side effects could be prevented (Shi, Expert Rev. Mol. Diagn. 1, 363-365 (2001)). It is obvious that developments which enable a time- and cost-efficient identification of nucleotide variations lead to further progress in pharmacogenetics.

SNPs account for the majority of genetic variations in the human genome and are the cause of more than 90% of the differences between individuals (Kwok, Annu. Rev. Genomics Hum. Genet 2, 235-258 (2001); Kwok und Chen, Curr. Issues Mol. Biol. 5, 43-60 (2003); Twyman and Primrose, Pharmacogenomics 4, 67-79 (2003)). To detect such genetic variations and other nucleic acid variants, such as mutations, various methods can be employed. For example, the identification of a variant of a target nucleic acid can be effected by hybridizing the nucleic acid sample to be analyzed with a hybridization probe specific for the sequence variant under suitable hybridization conditions (Guo et al., Nat. Biotechnol. 15, 331-335 (1997)).

- 2 -

However, it has been found that such hybridization methods fail to meet, in particular, the clinical requirements in terms of the necessary sensitivity of such assays. Therefore, especially PCR has also found broad use in molecular-biological and diagnostic examination methods for the detection of mutations, single-nucleotide polymorphisms (SNPs) and other allelic sequence variants (Saiki et al., Science 239, 487-490 (1988)), wherein a target nucleic acid to be examined in view of the existence of a variant is amplified by a polymerase chain reaction prior to hybridization. As hybridization probes for such assays, single-strand oligonucleotides are usually used. A modified embodiment of such assays includes those which employ fluorescent hybridization probes (Livak, Genet. Anal. 14, 143-149 (1999)). Generally, it is sought to automate methods for the determination of SNPs and other sequence variations (Gut, Hum. Mutat. 17, 475-492 (2001)).

An alternative of sequence variant specific hybridization which is already known in the prior art is offered by the so-called allele-specific amplification (Newton et al., Nucleic. Acids Res. 17, 2503-2516 (1989); Germer et al., genome res. 10, 258-266 (2000); Gibbs et al., Nucleic. Acids Res. 17, 2437-2448 (1989); Wu et al., PNAS 86, 2757-2769 (1989); Ishikawa et al., Hum. Immunol. 42, 315-318 (1995)). In this detection method, already during the amplification, variant-specific amplification primers are employed which usually have a so-called discriminating terminal nucleotide residue at the 3'-terminal end of the primer, which residue is merely complementary to only one specific variant of the target nucleic acid to be detected. In this method, nucleotide variations are determined by the presence or absence of DNA product after PCR amplification. The principle of allele-specific amplification is based on the formation of canonical or non-canonical primer-template complexes at the end of allele-specific primer probes. At a correctly paired 3' primer end, the amplification by a DNA polymerase can occur, while at a mismatched primer end, extension should be inhibited.

For example, U.S. 5,595,890 describes such methods for allele-specific amplification and their application for the detection of clinically relevant point mutations, for example, in the k-ras oncogene. U.S. 5,521,301 also describes methods for allele-specific amplification for the genotyping of the ABO blood

- 3 -

group system. In contrast, U.S. 5,639,611 discloses the use of allele-specific amplification in connection with the detection of the point mutation responsible for sickle-cell anemia.

However, allele-specific amplification is problematic in that it is characterized by a low selectivity, which necessitates further complicated and thus time- and cost-intensive optimization steps.

Such methods for detecting sequence variants, polymorphisms and mainly point mutations require allele-specific amplification especially when the sequence variant to be detected is deficient as compared with a predominant variant of the same nucleic acid segment (or of the same gene).

For example, such a situation occurs if disseminated tumor cells are to be detected in body fluids, such as blood, serum or plasma, by means of allele-specific amplification (U.S. 5,496,699). For this purpose, DNA is first isolated from body fluids such as blood, serum or plasma, which DNA is composed of a deficiency of DNA from disseminated tumor cells and an excess of DNA from non-proliferating cells. Thus, the mutations in the k-ras gene significant for tumoral DNA must be detected from a few copies of tumoral DNA in the presence of an excess of wild type DNA.

All the methods for allele-specific amplification described in the prior art have the disadvantage that, despite the use of 3'-discriminating nucleotide residues, a primer extension occurs to a lower extent in the presence of a suitable DNA polymerase even if the target nucleic acid does not exactly correspond to the sequence variant to be detected, i.e., is distinguished therefrom at least by the nucleotide complementary to the nucleotide residue to be discriminated. This leads to false-positive results especially if a particular sequence variant is to be detected in an excess background of nucleic acids containing another sequence variant. As mentioned above, this is the case, for example, in the detection of particular k-ras alleles as indicators of disseminated tumor cells. Another disadvantage of the known methods is the fact that a 3'-terminally discriminating oligonucleotide residue must be used at any rate. The main reason for the

- 4 -

disadvantages of these PCR-based methods is the incapability of the polymerases employed in these methods to sufficiently discriminate between base mismatches. Therefore, it has not yet been possible by PCR to directly obtain unambiguous information about the presence or absence of a mutation. To date, further time- and cost-intensive purification and analytical methods have always been necessary for an unambiguous diagnosis of such mutations. Therefore, novel methods which enable an enhancement of the selectivity of allele-specific PCR amplification will have a significant impact on the reliability and robustness of direct SNP analysis by PCR.

On the other hand, a number of modifications have already been described in the protein sequence of DNA polymerases I. Thus, U.S. Patent No. 6,329,178 mentions DNA polymerase mutants with altered catalytic activity in which there were mutations in the A motif (the highly conserved sequence DYSQIELR). In addition, Minnick, T. et al., J. Biol. Chem. 274, 3067-3075 (1999), describe a wide variety of *E. coli* DNA polymerase I (Klenow fragment) mutants in which alanine exchanges have been performed. Part of the mutants described exhibit a higher polymerase accuracy as compared to the wild type. One of the mutants mentioned is H881A; particular properties of these mutants with respect to the other mutants described are not stated.

Therefore, it was the object of the present invention to provide sequence variants with enhanced specificity by means of which a sequence variant specific detection method is enabled.

#### Summary of the invention

Surprisingly, it has been found that special mutants of family A DNA polymerases, namely those in which the conserved C motif and especially its QHV amino acid sequence has been modified, exhibit an enhanced mismatch discrimination and can be employed in detection methods for sequence variants. The thermostable variants thereof are suitable for allele-specific PCR. In detail, the present invention relates to:

- 5 -

- (1) a family A DNA polymerase which has a modified motif C sequence and an enhanced mismatch discrimination as compared to the corresponding wild type polymerase, or a Klenow fragment thereof;
- (2) a preferred DNA polymerase of embodiment (1), wherein in the motif C sequence QVH in positions 879-881, based on the *E. coli* DNA polymerase Klenow fragment shown in SEQ ID NO: 2, at least the amino acid residue Q879 has been replaced by a lipophilic amino acid residue;
- (3) a DNA sequence which encodes said DNA polymerase or the Klenow fragment thereof according to embodiment (1) or (2);
- (4) a vector which contains the DNA sequence according to embodiment (3);
- (5) a host cell which has been transformed with the vector according to embodiment (4) and/or includes a DNA according to embodiment (3);
- (6) a process for the preparation of a DNA polymerase or its Klenow fragment according to embodiment (1) or (2), which comprises culturing a host cell according to embodiment (5) and isolating the DNA polymerase or the Klenow fragment from the culture or the culture supernatant;
- (7) the use of the DNA polymerase or the Klenow fragment according to embodiment (1) or (2) in diagnostic and molecular-biological methods including allele-specific PCR, DNA amplification by means of PCR, cloning, etc.;
- (8) a method for determining the presence or absence of at least one sequence variant in one or more target nucleic acids in an individual sample using a DNA polymerase according to embodiment (1) or (2); and
- (9) a kit for determining the presence or absence of at least one sequence variant in one or more target nucleic acids in an individual sample according to the method of embodiment (8), containing at least one DNA polymerase according to embodiment (1) or (2).

- 6 -

Brief Description of the Figures

**Figure 1:** Autoradiographs after denaturing PAGE for examining the influence of mutations in *E. coli* DNA polymerase I (Klenow fragment, 5'→3' exonuclease-deficient) in positions 879-881 (SEQ ID NO: 2) on the selectivity of primer extension. Reactions contained 150 nM primer/template complex (primer: 5'-ACA AAA TAC CTG TAT TCC TX-3', X = A, G, C or T (SEQ ID NO: 11); template: 5'-GA TCC CTG GAC AGG CYA GGA ATA CAG GTA TTT TGT-3', Y = A, G, C or T (SEQ ID NO: 12), 1 mM each of dATP, dCTP, TTP, dGTP, and 600 nM DNA polymerase. Incubation at 37 °C for 10 min in buffer (50 mM Tris-HCl, pH 7.3, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 0.05% Triton® X-100).

**Figure 2:** Autoradiographs after denaturing PAGE for examining the influence of mutations in *E. coli* DNA polymerase I (Klenow fragment, 5'→3' exonuclease-deficient) in positions 879-881 (based on the Klenow fragment from *E. coli* as shown in SEQ ID NO: 2) on the selectivity of primer extension. Reactions contained 150 nM primer/template complex [**a:** primer: 5'-GAC CCA CTC CAT CGA GAT TTC T-3' (SEQ ID NO: 13); templates: 5'-GGT CTA GCT ACA GXG AAA TCT CGA TGG AGT GGG TC-3', X = A or T (SEQ ID NO: 14); **b:** primer: 5'-GTT TTA GAT GTT AAA TCA CAC TTA T-3' (SEQ ID NO: 15); template: 5'-CTT TCC AGA CAA CXT AAG TGT GAT TTA ACA TCT AAA AC-3', X = A or G (SEQ ID NO: 16)], 1 mM each of dATP, dCTP, TTP, dGTP, and 600 nM DNA polymerase. Incubation at 37 °C for 10 min in buffer (50 mM Tris-HCl, pH 7.3, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 0.05% Triton® X-100).

**Figure 3:** Autoradiographs after denaturing PAGE for examining the influence of mutations in the QVH motif in Taq DNA polymerase I on the selectivity of primer extension. Reactions contained 150 nM primer/template complex [**a:** primer: 5'-ACA AAA TAC CTG TAT TCC TX-3', X = T (SEQ ID NO: 11); template: 5'-GA TCC CTG GAC AGG CYA GGA ATA CAG GTA TTT TGT-3', Y = A or G (SEQ ID NO: 12); **b:** primer: 5'-GAC CCA CTC CAT CGA GAT TTC T-3' (SEQ ID NO: 13); template: 5'-GGT CTA GCT ACA GXG AAA TCT CGA TGG AGT GGG TC-3', X = A or T (SEQ ID NO: 14); **c:** primer: 5'-GTT TTA GAT GTT AAA TCA CAC TTA T-3' (SEQ ID NO: 15); template: 5'-CTT TCC AGA CAA CXT AAG TGT GAT TTA ACA

- 7 -

TCT AAA AC-3', X = A or G (SEQ ID NO: 16)], 1 mM each of dATP, dCTP, TTP, dGTP, and 0.6 ng of DNA polymerase. Incubation at 37 °C for 10 min in buffer (50 mM Tris-HCl (pH 9.2 at 25 °C), 16 mM ammonium sulfate, 2.5 mM MgCl<sub>2</sub>, 0.1 % Tween® 20).

Figure 4: Real time PCR experiments with Taq (wt) (SEQ ID NO: 4) and LVL mutant (SEQ ID NO: 4 with LVL in positions 782-784). The experiments were performed by means of an *iCycler* (BIORAD) system. A typical reaction in 20 µl contained: 40 pM of the respective template in Taq DNA polymerase buffer (50 mM Tris-HCl (pH 9.2 at 25 °C), 16 mM ammonium sulfate, 2.5 mM MgCl<sub>2</sub>, 0.1% Tween® 20, 0.3 mM dNTPs), 0.5 µM of the two primers and 95 ng of Taq DNA polymerase, and 1/50,000 of *SybrGreen I* 10,000x solution in DMSO (*Molecular Probes*). The PCR was performed with the following program: cycles at 95 °C for 30 s, 55 °C for 35 s, and 72 °C for 40 s. Reactions 1w, 2w, 3w and 4w were performed with the wild type enzyme, while 1m, 2m, 3m and 4m were performed with the LVL mutant. DNA sequences:

a: primer probe: 5'-d(GAC CCA CTC CAT CGA GAT TTC T) (SEQ ID NO: 19);  
reverse primer: 5'-d(AGA GGA AAG ATG AAG TAC TAT G) (SEQ ID NO: 20);  
template: 5'-d(CAA CTG TTC AAA CTG ATG GGA CCC ACT CCA TCG AGA TTT CX C TGT AGC TAG ACC AAA ATC ACC TAT TTT TAC TGT GAG GTC TTC ATG AAG AAA TAT ATC TGA GGT GTA GTA AGT AAA GGA AAA CAG TAG ATC TCA TTT TCC TAT CAG AGC AAG CAT TAT GAA GAG TTT AGG TAA GAG ATC TAA TTT CTA TAA TTC TGT AAT ATA ATA TTC TTT AAA ACA TAG TAC TTC ATC TTT CCT CT), X = A (wild type) (1) or T (mutant) (2) (SEQ ID NO: 21).

b: primer probe: 5'-d(GTT TTA GAT GT TAA ATC ACA CTT AT) (SEQ ID NO: 22);  
reverse primer: 5'-d(AAA GCT CCT TTC TGA ATA TTG AG) (SEQ ID NO: 23);  
template: 5'-d(AAA ATG TGA GAA GGG ACC TCA TAA AAT ATG TCA TAT GGA AAT GAG CAG ATA ATA AAG ATT ATA GCT TTT CTT TGT CAA AAG GAG ACT CAA TAT CTT TAC TCT TTC ATC AGG ACA TTG TGA CAA ATG TTT CCC CCA GAA TCA TCC GGG GAA CCA CCT CTG GCC CCA TGT ATG GCC CTG GAC AAA GCT CCT TTC TGA ATA TTG AGC TCA TCA GTG AGA AAA CGG CTG CAT ATT GGT GTC AAA GTG TCA CTG AAC TAA AGG CTG ACT TTC CAG ACA ACX TAA GTG TGA TTT AAC ATC TAA AAC), X = A (wild type) (3) or G (mutant) (4) (SEQ ID NO: 24).

- 8 -

**Figure 5:** Autoradiographs after denaturing PAGE for a comparative examination of the influence of mutations in *E. coli* DNA polymerase I (Klenow fragment, 5'→3' exonuclease-deficient) in positions 879-881 (SEQ ID NO: 2) on the selectivity of primer extension. The mutant LVL according to the invention was compared with the mutant QVA known from the literature (Minnick, T. et al., J. Biol. Chem. 274, 3067-3075 (1999)). Reactions contained 150 nM primer/template complex (primer: 5'-ACA AAA TAC CTG TAT TCC TX-3', X = A, G, C or T (SEQ ID NO: 11); template: 5'-GA TCC CTG GAC AGG CYA GGA ATA CAG GTA TTT TGT-3', Y = A, G, C or T (SEQ ID NO: 12), 1 mM each of dATP, dCTP, TTP, dGTP, and 600 nM DNA polymerase. Incubation at 37 °C for 10 min in buffer (50 mM Tris-HCl, pH 7.3, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 0.05% Triton® X-100).

Sequence listing - free text

SEQ ID NO:	Description - free text
1	<i>E. coli</i> wild type Klenow fragment of DNA polymerase 1
2	<i>E. coli</i> Klenow fragment of DNA polymerase 1
3	wild type Taq polymerase
4	wild type Taq polymerase
5	primer
6	downstream primer
7	antisense primer
8	primer FVL20TH
9	template TFVL90A
10	template TFVL90G
11	primer for the detection of SNP in the human genomic factor V Leiden DNA sequence
12	template of the human genomic factor V Leiden DNA sequence; n = g, wild type template; n = a, mutant template
13	primer for the detection of human somatic BRAF-T1796A mutation
14	wild type template of the BRAF gene; w = t, wild type template; w = a, mutant template
15	primer for the detection of human dihydropyrimidine dehydrogenase (DPyD) mutation G735A
16	template of the human DPyD; r = g, wild type template; r = a, mutant template
17	primer for the detection of human acid ceramidase mutation A107G



- 9 -

18	template of human acid ceramidase; r = a, wild type template; r = g, mutant template
19	primer probe BrafT
20	reverse primer for BRAF
21	target template BrafX; w = a, Braf A (wild type); w = t, BrafT (mutant)
22	primer probe DpyDT
23	reverse primer for DpyDT
24	target template DpyDX; r = a, DpyDA (wild type); r = t, DpyDT (mutant)
25	pTTQ18::Taq
26	pQE30
27	template segment (SEQ ID NO: 14); w = t, wild type; w = a, mutant template
28	template segment (SEQ ID NO: 16); r = g, wild type; r = a, mutant
29	template segment (SEQ ID NO: 12); r = t, wild type; r = a, mutant

#### Detailed description of the invention

The present invention relates to mutant family A DNA polymerases having an enhanced capability of mismatch discrimination, or Klenow fragments thereof. The enhanced capability of mismatch discrimination, which means a high selectivity according to the Watson-Crick rules during the incorporation of complementary bases and also already during the annealing of a primer to the template, i.e., a higher extension selectivity ratio ( $F_{\text{match}}/F_{\text{mismatch}}$ ) as compared to the corresponding starting polymerase (wild type) (e.g., determined in a fluorescence test system according to Example 2), can be achieved by mutating a particular amino acid sequence in natural enzymes. The properties of DNA polymerases caused thereby are superior to those of the "state of the art" wild type polymerases as currently sold commercially. The enhancement of the selectivity of the activity of DNA polymerases enables more reliable systems for detecting mutations or polymorphisms, a direct diagnosis by allele-specific PCR without downstream time- and cost-intensive purification and analytical methods, and a high sustainability because no chemically modified primers need to be used.

The following definitions are to be applied to the whole application, they are however not to be construed as limiting the invention. "Family A DNA polymer-

- 10 -

ases" (also referred to as "polymerases I") are those DNA-polymerizing enzymes which contain the A motif with the sequence DYSQIELR in their active site. They also include the enzymes described herein which have mutations in the C motif. In particular, they also include thermostable DNA polymerases and their mutants.

The term "Klenow fragment" as used herein means any C-terminal fragment of a family A DNA polymerase which has both polymerase activity and 3'→5' exonuclease activity (but no 5'→3' exonuclease activity). "Vectors" as used herein include plasmids, cosmids, phagemids and viruses which, in addition of a DNA of interest (which are, in particular, sequences of family A DNA polymerases according to the invention), also include regulation elements which control the expression of the DNA of interest.

The term "host cells" includes both prokaryotic cells, such as *E. coli* (especially *E. coli* XL1-blue, DH5 $\alpha$ , BI21 (DE3), M15 [pREP4], SG13005 [pREP4], BL21 (DE3) pLysS), *Halomonas elongata*, *Caulobacter sp.*, *Halobacterium halobium* etc., and eukaryotic cells, such as yeast and other fungal cells, plant and animal cells including isolated human cells in cell culture. Further, the term "host cell" also relates to cell extracts which, when presented an mRNA, are able to translate it, such as wheat germ extract and rabbit reticulocyte extract (RRL). Further, "host cell" also includes *in vitro* expression systems, such as T7 Expression System, pBAD Expression System, ThioFusion™ Expression Systems, trc Expression System, PL Expression System, PurePro™ *Caulobacter* Expression System, Microbiological Media and Media Components, Qiagen pQE Expression System and Novagen pET Expression System, etc.

Embodiments (1) and (2) of the invention relate to family A DNA polymerases or their Klenow fragment which are distinguished from naturally occurring DNA polymerases by having an enhanced mismatch discrimination, which results in an increased selectivity of enzyme activity. The DNA polymerases according to the invention are derived from bacterial DNA polymerases, such as polymerases from *E. coli*, *Aquifex*, *Borrelia*, *Bacillus*, *Chlamydia*, *Chlamydophila*, *Chloroflexus*, *Haemophilus*, *Heliobacter*, *Lactococcus*, *Methylobacterium*, *Mycobacterium*, *Rhodothermus*, *Rickettsia*, *Streptococcus*, *Streptomyces*, *Synechocystis*, *Tre-*

- 11 -

*ponema* etc., but also, in particular, from polymerases of thermostable organisms, such as *Thermus aquaticus*, *Thermus thermophilus*, *Thermus filiformis*, *Rhodothermus obamensis*, *Bacillus stearothermophilus* etc. In particular, in the DNA polymerases according to embodiment (1) of the invention or their Klenow fragments, at least one amino acid residue, preferably Q and/or H, in the motif C sequence QVH in positions 879-881 (based on the *E. coli* DNA polymerase Klenow fragment shown in SEQ ID NO: 2) has been replaced by a lipophilic amino acid residue.

In embodiment (2) of the invention, at least the amino acid residue Q879, based on SEQ ID NO: 2, has been replaced by a lipophilic amino acid residue. In a preferred aspect of embodiment (2), the amino acid residue H881 in the motif C sequence QVH has been further replaced by a lipophilic amino acid residue.

In a further preferred aspect of embodiments (1) and (2), the amino acid residue in position 880 (based on SEQ ID NO: 2) is selected from Val, Leu, Ile, Ala and Tyr, more preferably from Val and Ile.

"Lipophilic amino acid residues" within the meaning of the present invention comprise the amino acid residues Gly, Ala, Val, Leu, Met, Phe, Trp, Ile, Pro etc. Preferred residues are Gly, Ala, Val, Leu and Ile. According to the present invention, the motif C sequence QVH has been preferably replaced by the sequences LVL, LVG, QVL, PIL, QVV, LVA, LAA, LVV, LVI, IVI, III, VVV, QVV, QVA etc., replacement by LVL and LVG being particularly preferred.

In addition to the above mentioned exchange, the polymerase according to the invention can include more mutations, such as deletions, substitutions and/or additions (up to 20 amino acid residues each), provided that the higher extension ratio ( $F_{\text{match}}/F_{\text{mismatch}}$ ) as compared to the wild type is not adversely affected thereby. The substitutions include, in particular, further (preferably conservative) exchanges in the motif C sequence, which are effected in addition to the above stated replacing of at least one residue in QVH by a lipophilic amino acid residue. Thus, the present invention also includes those DNA polymerases, in

- 12 -

particular, which contain an amino acid sequence LVN, LYH, PLQ, LVQ, QDL, QEL, QUV etc. instead of QVH in the C motif.

In addition, it has been found that a DNA polymerase with the sequence QVN instead of QVH in the C motif also has a higher extension ratio ( $F_{\text{match}}/F_{\text{mismatch}}$ ) as compared to the wild type.

Further, the invention includes Taq polymerase whose QVH sequence has been exchanged as described above. Particularly preferred are those Taq polymerases whose QVH sequence has been replaced by LVL or LVG (based on the Taq polymerase protein sequence shown in SEQ ID NO: 4, the positions 782-784 are affected by the exchange).

In the Klenow fragments according to the invention, it is preferred that at least two amino acid residues in QVH have been replaced by lipophilic amino acid residues. Particularly preferred among the sequences with two exchanges as mentioned above are LVL and LVG, also for the Klenow fragments.

As compared to the mutant QVA known from the literature (Minnick, T. et al., J. Biol. Chem. 274, 3067-3075 (1999)), the polymerase mutants according to the invention have an increased selectivity of primer extension (Example 7, Figure 5). As shown in Figure 5, the QVA mutant has an essentially higher tendency to extend mismatches as compared to the LVL mutant according to the invention.

Thus, the amino acid sequence of the DNA polymerases according to the invention has been altered in one or more positions as compared to the wild type DNA polymerases, and this is also reflected on the nucleic acid level. The invention also relates to a DNA sequence which codes for one of the DNA polymerases according to the invention or its Klenow fragment (embodiment (3) of the invention). The invention also relates to a vector which contains the DNA sequence coding for the DNA polymerase (1) or (2), and to a host cell transformed with a vector which contains the DNA sequence coding for the DNA polymerase (1) or (2), and/or which includes a DNA coding for the DNA polymerase (1) or (2). The invention also relates to a process for preparing a DNA polymerase or its Klenow fragment, which

- 13 -

comprises culturing the transformed host cell and isolating the DNA polymerase or the Klenow fragment from the culture or the culture supernatant.

Thermostable DNA polymerases according to embodiment (1) or (2) of the invention have a higher selectivity in the polymerase chain reaction (PCR) and discriminate better between individual mismatches and canonic complexes as compared to naturally occurring DNA polymerases. This leads to improved properties of the mutants when used in diagnostic (allele-specific PCR) and molecular-biological (DNA amplification by PCR, cloning) methods. Therefore, the invention also relates to methods in which the DNA polymerase according to the invention or its Klenow fragment can be employed.

The DNA polymerase according to embodiment (1) or (2) of the invention can also be employed in a method for determining the presence or absence of sequence variants in one or more target nucleic acids in an individual sample (embodiment (8)). Such a method preferably comprises one or more of the following steps:

a) adding:

- deoxynucleoside triphosphates;
- one of the DNA polymerases according to the invention;
- at least one discriminating primer containing at least one discriminating nucleotide residue, wherein a primer is added for each sequence variant of a target nucleic acid to be detected, which primer has a sequence complementary to the sequence variant to be detected, and wherein the sequence variant to be detected in the target nucleic acid is complementary to at least one 3'-terminal, 3'-proxi-terminal or 3'-proxi-proxi-terminal nucleotide residue of the discriminating primer;
- at least one other primer which is complementary to a primer extension product formed by extension of a discriminating primer;

- 14 -

- b) performing a primer extension reaction wherein an extension product of the discriminating primer is obtained substantially only if the sample contains a target nucleic acid with the sequence variant to be detected;
- c) separating the product of the primer extension reaction from the template nucleic acid;
- d) reiterating steps b) and c) to obtain an amplification product, for example, by polymerase chain reaction;
- e) determining the presence or absence of a sequence variant from the presence or absence of the amplification product.

In this connection, the terms used for the description of the method according to the invention have the following meanings:

A "DNA polymerase according to the invention" is a family A DNA polymerase as defined above which includes the A motif with the sequence DYSQIELR in its active site and comprises particular mutations in the C motif. In particular, they also include thermostable DNA polymerases with mutations in the C motif. These mutations are conservative substitutions of the QVL amino acid residues of the C motif and/or the above defined non-conservative substitutions.

A "thermostable DNA polymerase" is a polymerase which is functional even at temperatures of above 42 °C and can be employed, in particular, in PCR-based amplification methods.

In particular, the term "extension reactions" includes reaction mixtures which comprise at least a polymerase, nucleotides, one or more templates and primers. The reaction conditions are selected in such a way that the primer(s) can anneal to the template, and the polymerase catalyzes the extension of the primer(s) by incorporating nucleotides complementary to the template. The product formed is a primer extension product.

- 15 -

A "target nucleic acid" is a nucleic acid segment from a biological sample whose sequence is to be analyzed further by means of the method according to the invention. The biological sample usually consists of genomic DNA. However, the method according to the invention is similarly suitable for analyzing RNA sequence variants. It is not critical whether the sample was isolated from cellular material or from biological fluids, such as blood, serum, plasma, urine or saliva.

A "sequence variant" according to the invention means a target nucleic acid with a particular nucleic acid sequence which exhibits only minute differences from the sequences of other possible target nucleic acids and can be identified by these minute differences. The differences in sequence preferably affect from one to three contiguous nucleotide residues. The present invention is particularly suitable for the identification of sequence variants relating to a single nucleotide residue (SNP). This may be a base exchange, but alternatively, it may also be nucleotide additions or deletions. In this way, different alleles can be distinguished from one another. Point mutations or polymorphisms can also be detected in this way. Thus, in particular, a "sequence variant" also includes point mutations and polymorphisms which are analyzed with respect to prognostic or diagnostic issues.

In a template-directed polymerization of deoxynucleotide triphosphates, a polymerization of the deoxynucleotide triphosphates is effected from the 3' end of a so-called primer, which is hybridized to a single-strand template nucleic acid, to form a sequence complementary to the target nucleic acid. Such polymerization reactions in 5'→3' orientation are preferably performed enzymatically with so-called DNA polymerases, such as Klenow polymerase. Particularly preferred are thermostable DNA polymerases, such as Taq polymerase (Roche Applied Science Catalogue No. 1146165).

A "discriminating primer" within the meaning of the invention is a primer whose sequence is exactly complementary to a particular sequence variant, this sequence having particular differences from another sequence variant which may be present in the sample to be analyzed. In this connection, a "discriminating nucleotide residue" means a nucleotide residue whose complement is formed by different nucleotide residues in the different existing sequence variants.

- 16 -

The "3' terminal nucleotide residue" is the nucleotide residue which is positioned at that terminal end of an oligonucleotide primer which has a free 3'-OH group. The "proxi-terminal nucleotide residue" is the nucleotide residue of an oligonucleotide primer whose end is linked to the 5'-end of the terminal nucleotide residue through a phosphate group. "Proxi-proxi-terminal nucleotide residue" refers to the nucleotide residue whose 3'-end is linked to the 5'-end of the proxi-terminal nucleotide residue through a phosphate group.

As can be seen from the above description of the method according to the invention, the steps a) to e) are essentially an amplification reaction which does or does not result in an amplification product depending on the presence or absence of a particular sequence reaction. Therefore, such methods can be performed according to protocols for PCR reactions known from the prior art.

In this connection, the invention also relates to a kit containing agents for performing the method according to the invention. In particular, such a kit contains a DNA polymerase according to the invention. Optionally, such a kit may contain additional components, such as one or more (discriminating) primers, deoxynucleotide triphosphate, buffers, quantification reagents, especially intercalating reagents, or reagents binding to the minor groove, wherein more preferably reagents from the group consisting of PicoGreen (Molecular Probes), SybrGreen (Molecular Probes), ethidium bromide, Gelstar (Cambrex) and Vista Green (Amersham) are selected, polymerase-blocking antibodies, especially TaqBlock, and agents for the template-directed polymerization of the deoxynucleotide triphosphates. In various embodiments, the individual components of the kit can be alternatively contained either together in one storage container or separately in two or more storage containers.

As can be seen from the Examples stated below, the observable effects in terms of improvement of specificity over the methods available from the prior art can be demonstrated clearly in a quantitative way (cf. Example 7, Figure 5) and have the result that under PCR amplification conditions, extension products of sequence variant specific primers indeed can be obtained essentially only if the sample to be analyzed contains a target nucleic acid with the sequence variant to be detected.



- 17 -

This specific effect can be optimized by improving and optimizing the respective PCR parameters by means of measures known to the skilled person from the prior art, and adapted to the sequence variant to be respectively detected.

Another embodiment of the present invention relates to the performance of the method according to the invention by means of real time PCR. In this method, the end products of the amplification reaction are not detected by gel electrophoresis, but the course of the amplification reaction is traced by means of suitable fluorescence-labeled hybridization probes, so that kinetic real-time measurements and quantifications are possible.

The hybridization probes to be employed for the methods according to the invention are usually single-stranded nucleic acids, such as single-stranded DNA or RNA or their derivatives, or alternatively, PNAs which hybridize to the target nucleic acid at the annealing temperature of the amplification reaction. Usually, these oligonucleotides have a length of from 20 to 100 nucleotides.

Depending on the precise detection format, the labeling can be introduced at any ribose or phosphate group of the oligonucleotide. Labels at the 5' and 3' end of the nucleic acid molecule are preferred. The type of labeling must be detectable in the real-time mode of the amplification reaction. This is possible, for example, not only with fluorescence labels, but alternatively also by means of labels which are detectable by NMR.

Many different test set-ups are possible. The following three detection format have proven particularly suitable in connection with the present invention:

1. FRET hybridization probes: For this test format, two single-stranded hybridization probes which are complementary to neighboring sites of the same strand of the amplified target nucleic acid are used simultaneously. Both probes have been labeled with different fluorescence components. Upon excitation of a first component with light of a suitable wavelength, it transfers the absorbed energy to the second component according to the principle of fluorescence resonance energy transfer, so that, when both hybridization samples bind to neighboring positions of

- 18 -

the target molecule to be detected, a fluorescence emission of the second component can be measured.

Alternatively, a fluorescence-labeled primer and only one labeled oligonucleotide probe can be used (Bernard et al., Analytical Biochemistry 235, 1001-107 (1998)).

2. TaqMan hybridization probes: A single-stranded hybridization probe is labeled with 2 components. When the first component is excited with light of a suitable wavelength, the absorbed energy is transferred to the second component, the so-called quencher, according to the principle of fluorescence resonance energy transfer. During the annealing step of the PCR reaction, the hybridization probe binds to the target DNA and is degraded by the 5'→3' exonuclease activity of the Taq polymerase during the subsequent elongation phase. Thus, the excited fluorescence component and the quencher are separated in space from each other, so that a fluorescence emission of the first component can be measured.

3. Molecular Beacons: These hybridization probes are also labeled with a first component and a quencher, the labels preferably being positioned at the two ends of the probe. In solution, the two components are in close spatial proximity due to the secondary structure of the probe. After hybridization to the target nucleic acid, both components are separated from one another, so that, after excitation with light of a suitable wavelength, the fluorescence emission of the first component can be measured (Lizardi et al., US 5,118,801).

In alternative embodiments, the respective amplification product can also be detected by a DNA binding dye according to the invention, which upon interaction with a double-stranded nucleic acid emits a corresponding fluorescence signal upon excitation with light of a suitable wavelength. The dyes SybrGreen and SybrGold (Molecular Probes) have proven particularly suitable for this application. Alternatively, intercalating dyes may also be used.

The invention is further illustrated by means of the following Examples and Figures. The procedures described are not to be considered limitations of the

- 19 -

invention, but mere examples which, even after modification, still describe the subject matter of the invention.

### Examples

#### Example 1: Construction of a library and purification of Klenow fragment variants

The plasmid pQKF<sup>-</sup> (Brakman, S., Nieckchen, P., ChemBioChem 2001, 2, 773-7; see also the equivalent plasmid pQE30 shown in SEQ ID NO: 26) enables the expression of the N-terminal 6-his-labeled Klenow fragment of *E. coli* DNA polymerase I (3'→5' exo<sup>-</sup>) under the control of a T5 promoter/Double lac operator sequence. The introduction of mutations into the motif C sequence which codes for Q879, V880 and H881 was effected by a two-step megaprimer mutagenesis. PCR reactions were performed by using PfuTurbo DNA polymerase (Stratagene) and under standard conditions. The first PCR was performed with a doped primer library (5'-GTA CGT ATG ATC ATG NNN NNN NNN GAT GAA CTG GTA TTT-3'; SEQ ID NO: 5) which was constructed in such a way as to contain 40% non-wild type nucleotides on each of the nine target positions and a 23mer downstream primer (5'-GCT AAT TAA GCT TGG CTG CAG GC-3'; SEQ ID NO: 6), which yielded a 195mer PCR product. The second PCR was performed by using the 195mer purified on agarose gel and a 24mer antisense primer (5'-TAC ATG GAC CTT TAC TTC GAA CGC-3' SEQ ID NO: 7) and yielded a 457 bp product which was digested with Csp45I and HindIII and cloned into pQKF<sup>-</sup>. The resulting plasmid library was transformed into *E. coli* XL 1blue (Stratagene), clones were selected from agar plates and separately grown over night in 96-well plates containing Superbroth medium (100 µg/ml ampicillin). Klenow fragment variants were expressed, harvested and lysed in 600 µl cultures using 96-well plates as described. The 300 µl lysates obtained were diluted with 900 µl of storage buffer (50 mM Tris-HCl, pH 7.3, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 1 µg/ml leupeptin and 1 µg/ml aprotinin), centrifuged and stored at -80 °C.

For primer extension reactions and steady state kinetic measurements, the Klenow fragment and selected mutants were expressed as described above, and purified

- 20 -

by means of Ni-NTA agarose (Qiagen) following the manufacturer's instructions, but omitting the imidazole in the lysing and washing steps. The enzymes obtained were >95% pure, which was confirmed by SDS PAGE with Coomassie blue staining. After replacing the buffer (by 100 mM  $K_2HPO_4$ , 1 mM DTT, pH 6.5, with 50% glycerol), concentrations were measured using the nanoOrange assay (Molecular Probes) and adjusted to 1  $\mu$ g/ $\mu$ l.

#### Example 2: Screening

The reaction mixtures for screening the library contained 150 nM template, 225 nM primer, 50 mM Tris-HCl, pH 7.3, 10 mM  $MgCl_2$ , 1 mM DTT, 0.05% Triton<sup>®</sup> X-100 and 200  $\mu$ M each of dNTPs. The reactions comprised the 20mer primer FVL20TH (5'-ACA AAA TAC CTG TAT TCC TT-3'; SEQ ID NO: 8) which is designed to bind with its 3'-terminal base to the human SNP G1691A, which is involved in the factor V Leiden mutation. For measurements of pairing extension efficiencies, the 90mer template TFVL90A (5'-GAC ATC ATG AGA GAC ATC GCC TCT GGG CTA ATA GGA CTA CTT CTA ATC TGT AAG AGC AGA TCC CTG GAC AGG CAA GGA ATA CAG GTA TTT-3'; SEQ ID NO: 9) which codes for the mutant allele 1691A of the human factor V-ORF was used, which resulted in a TA base pair at the 3' terminus of the primer. To obtain access to activities of Klenow variants which process a mismatched primer terminus, the 90mer template TFVL90G (5'-GAC ATC ATG AGA GAC ATC GCC TCT GGG CTA ATA GGA CTA CTT CTA ATC TGT AAG AGC AGA TCC CTG GAC AGG CGA GGA ATA CAG GTA TTT-3'; SEQ ID NO: 10) which codes for the corresponding wild type allele 1691G was used, which resulted in a TG mismatch at the 3' primer terminus. Both reactions were performed in parallel for each element of the library to enable an evaluation of the activity ratios as extension selectivities. Ten  $\mu$ l of the reaction mixtures was dispensed into black 384-well plates preheated at 37 °C using an automated liquid handling device (Hamilton Microlab Star), followed by adding 5  $\mu$ l of lysate solution. After 10 min, the reactions were stopped by adding 30  $\mu$ l of stopping solution (50 mM Tris-HCl, pH 7.3, 100 mM NaCl, 10 mM EDTA) which contained 3.4x SYBRGreen I (Molecular Probes) for quantifying the dsDNA produced by Klenow variants. The fluorescence intensities were quantified by means of a fluorescence plate reader (Polarstar Optima, BMG Labtechnologies

- 21 -

GmbH) with excitation at 485 nm and emission at 520 nm. The ratios of the measured fluorescence intensities ( $F_{\text{match}}/F_{\text{mismatch}}$ , arbitrary units) were employed for determining the extension selectivity. All DNA polymerases with a higher extension selectivity ratio ( $F_{\text{match}}/F_{\text{mismatch}}$ ) than that of the wild type were identified as enzymes having an increased extension selectivity.

### Example 3: Primer extension assays

Primer-template substrates were annealed by mixing 5'-<sup>32</sup>P-labeled primer in a specific reaction buffer (50 mM Tris-HCl, pH 7.3, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 0.05% Triton® X-100) with a twofold amount of template. The mixture was heated at 95 °C for 5 min and subsequently allowed to cool to room temperature over 1 hour. After the annealing, dNTPs were added, and the solution was incubated at 37 °C for 5 min. 15 µl reactions were initiated by adding 5 µl of enzyme solution in 1x reaction buffer to 10 µl of annealing mixture, followed by incubation at 37 °C for 10 min. The assays included 150 nM primer, 225 nM template, 1 mM each of dNTPs and 590 nM enzyme in a suitable reaction buffer. After 10 min of incubation, the reactions were stopped by adding 30 µl of gel-loading buffer (80% formamide, EDTA, 20 mM), and the product mixtures were analyzed by 14% denaturing PAGE (see Figures 1 and 2). The following primer and template sequences were employed in connection with different SNPs (positions underlined):

Human genomic factor V Leiden DNA sequence: Primer: 5'-ACA AAA TAC CTG TAT TCC TT-3' (SEQ ID NO: 11), wild type template: 5'-GAT CCC TGG ACA GGC GAG GAA TAC AGG TAT TTT GT-3' (SEQ ID NO: 12), mutant template: 5'-GAT CCC TGG ACA GGC AAG GAA TAC AGG TAT TTT GT-3' (SEQ ID NO: 12).

Human somatic BRAF-T1796A mutation: Primer: 5'-GAC CCA CTC CAT CGA GAT TTC T-3' (SEQ ID NO: 13), wild type template: 5'-GGT CTA GCT ACA GTG AAA TCT CGA TGG AGT GGG TC-3' (SEQ ID NO: 14), mutant template: 5'-GGT CTA GCT ACA GAG AAA TCT CGA TGG AGT GGG TC-3' (SEQ ID NO: 14).

- 22 -

Human dihydropyrimidine dehydrogenase (DPyD) mutation G735A: Primer: 5'-GTT TTA GAT GTT AAA TCA CAC TTA T-3' (SEQ ID NO: 15), wild type template: 5'-CTT TCC AGA CAA CGT AAG TGT GAT TTA ACA TCT AAA AC-3' (SEQ ID NO: 16), mutant template: 5'-CTT TCC AGA CAA CAT AAG TGT GAT TTA ACA TCT AAA AC-3' (SEQ ID NO: 16).

Human acid ceramidase mutation A107G: Primer: 5'-CGT TGG TCC TGA AGG AGG AT-3' (SEQ ID NO: 17), wild type template: 5'-AAA TCA ACC TAT CCT CCT TCA GGA CCA ACG TAC-3' (SEQ ID NO: 18), mutant template: 5'-AAA TCA ACC TGT CCT CCT TCA GGA CCA ACG TAC-3' (SEQ ID NO: 18).

Thus, the mutations in *E. coli* DNA polymerase I (Klenow fragment, 5'→3'-exonuclease-deficient) with LVL and LVG in positions 879-881 (based on the Klenow fragment from *E. coli* as shown in SEQ ID NO: 2) as compared to the wild type enzyme with QVH in positions 879-881 (Figs. 1 and 2).

#### Example 4: Cloning of Taq DNA polymerase

The plasmid pTTQ18::Taq (SEQ ID NO: 25) was constructed by Engelke *et al.* (Anal. Biochem. 1990, 191, 396-400 (1990)) and enables the expression of Taq DNA polymerase under the control of a *Ptac* promoter/*lac* operator sequence. The LVL mutation was introduced into the Taq QVH motif by PCR using the Stratagene QuikChange® Kit. The resulting mutant plasmid and the wild type plasmid were transformed into *E. coli* XL1 Blue (Stratagene). Clones were selected and grown over night in 20 ml of Superbroth (100 µg/ml carbenicillin). The expression of the Taq clones was performed in cultures in 1 l of Superbroth (100 µg/ml carbenicillin), and the cells were harvested after 16 h of induction with 1 mM IPTG. The purification of the Taq DNA polymerases was performed as described by Engelke *et al.* (Anal. Biochem. 1990, 191, 396-400). Instead of purification by ion-exchange, gel filtration using a column with Sephadex® 75 (Amersham) was applied. The enzymes obtained were > 90% pure, which was confirmed by SDS PAGE with Coomassie blue staining. The concentrations were measured using the nanoOrange assay (Molecular Probes) and SDS PAGE with Coomassie blue staining.

- 23 -

Example 5: Primer extension with catalysis by Taq DNA polymerase

Primer-template substrates were annealed by mixing 5'-<sup>32</sup>P-labeled primer in specific reaction buffer (50 mM Tris-HCl, pH 9.2 at 25 °C, 16 mM ammonium sulfate and 2.5 mM MgCl<sub>2</sub>, 0.1% Tween<sup>®</sup> 20) with a twofold amount of template. The mixture was heated at 95 °C for 10 min and subsequently allowed to cool to room temperature over 1 hour. After the annealing, dNTPs were added, and the solution was incubated at 37 °C for 5 min. 15 µl reactions were initiated by adding 5 µl of enzyme solution in 1x reaction buffer to 10 µl of annealing mixture, followed by incubation at 72 °C for 10 min. The assays included 150 nM primer, 225 nM template, 1 mM each of dNTPs and 0.5 ng of Taq LVL DNA polymerase (mutant polymerase) and 0.06 ng of Taq DNA polymerase in a suitable reaction buffer. After 10 min of incubation, the reactions were stopped by adding 30 µl of gel-loading buffer (80% formamide, EDTA, 20 mM), and the product mixtures were analyzed by 14% denaturing PAGE (see Figure 3). As to the primer and template sequences used in connection with the SNPs human genomic factor V Leiden DNA sequence, human somatic BRAF-T1796A mutation and human dihydropyrimidine dehydrogenase (DPyD) mutation G735A, reference is made to Example 3.

Example 6: Real-time PCR experiments

Real-time PCR was performed using an *iCycler* system (BIORAD). The reactions were performed in a total volume of 20 µl which contained 4 pM of the respective templates in Taq DNA polymerase buffer (50 mM Tris-HCl, pH 9.2 at 25 °C, 16 mM ammonium sulfate and 2.5 mM MgCl<sub>2</sub>, 0.1% Tween<sup>®</sup> 20. The final mixtures contained dNTPs (200 µM each of dATP, dGTP, dCTP and TTP), primers (0.5 µM each of the respective primer probe and the reverse primer) and 13 ng of Taq DNA polymerase (SEQ ID NO: 4), 95 ng of DNA polymerase from Taq LVL mutant (SEQ ID NO: 4 with LVL in positions 782-784) and an aqueous 1/50,000 dilution of a 10,000 fold solution of *SybrGreen I* in DMSO (*Molecular Probes*). All PCR amplifications were performed using the following program: Initial denaturing at 95 °C for 3 min, followed by 40 cycles of denaturing at 95 °C for 30 s, primer annealing at 55 °C for 35 s and extension at 72 °C for 40 s. The results

- 24 -

presented are derived from independent triplicate measurements repeated at least three times and issued from a parent mixture. The results are summarized in Figure 4. The following DNA sequences were employed:

Sequences in connection with BRAF: Primer probe BraFT: 5'-d(GAC CCA CTC CAT CGA GAT TTC T) (SEQ ID NO: 19), reverse primer: 5'-d(AGA GGA AAG ATG AAG TAC TAT G) (SEQ ID NO: 20), target template BraFX: 5'-d(CAA CTG TTC AAA CTG ATG GGA CCC ACT CCA TCG AGA TTT CXC TGT AGC TAG ACC AAA ATC ACC TAT TTT TAC TGT GAG GTC TTC ATG AAG AAA TAT ATC TGA GGT GTA GTA AGT AAA GGA AAA CAG TAG ATC TCA TTT TCC TAT CAG AGC AAG CAT TAT GAA GAG TTT AGG TAA GAG ATC TAA TTT CTA TAA TTC TGT AAT ATA ATA TTC TTT AAA ACA TAG TAC TTC ATC TTT CCT CT), X = A, BraFA, X = T, BraFT (SEQ ID NO: 21). Sequences in connection with DPyD: Primer probe DpyDT: 5'-d(GTT TTA GAT GT TAA ATC ACA CTT AT) (SEQ ID NO: 22), reverse primer: (5'-d(AAA GCT CCT TTC TGA ATA TTG AG) (SEQ ID NO: 23), target template DPyDX: 5'-d(AAA ATG TGA GAA GGG ACC TCA TAA AAT ATG TCA TAT GGA AAT GAG CAG ATA ATA AAG ATT ATA GCT TTT CTT TGT CAA AAG GAG ACT CAA TAT CTT TAC TCT TTC ATC AGG ACA TTG TGA CAA ATG TTT CCC CCA GAA TCA TCC GGG GAA CCA CCT CTG GCC CCA TGT ATG GCC CTG GAC AAA GCT CCT TTC TGA ATA TTG AGC TCA TCA GTG AGA AAA CGG CTG CAT ATT GGT GTC AAA GTG TCA CTG AAC TAA AGG CTG ACT TTC CAG ACA ACX TAA GTG TGA TTT AAC ATC TAA AAC), X = A DpyDA, X = T, DpyDG (SEQ ID NO: 24). The oligonucleotides BraFX and DpyDX (SEQ ID NOS: 21 and 24) were synthesized and purified by IBA, Göttingen, Germany.

#### Example 7 (Comparative Example): Comparison of mismatch discrimination

A mutant according to the invention and a mutant known from the literature of *E. coli* DNA polymerase I (Klenow fragment, 5'→3'-exonuclease-deficient) in positions 879-881 (SEQ ID NO: 2) were compared for their selectivity of primer extension. The mutants employed were the LVL mutant (SEQ ID NO: 2 with LVL in positions 879-881) and the QVA mutant which corresponds to the Klenow fragment with H881A from Minnick, T. et al., J. Biol. Chem. 274, 3067-3075 (1999) (SEQ ID NO: 2 with HVA in positions 879-881).



- 25 -

With the two mutants mentioned, primer extension assays according to Example 3 were performed. The mismatch discrimination with the human genomic factor V Leiden DNA sequence was tested: Primer: 5'-ACA AAA TAC CTG TAT TCC TT-3' (SEQ ID NO: 11), wild type template: 5'-GAT CCC TGG ACA GGC GAG GAA TAC AGG TAT TTT GT-3' (SEQ ID NO: 12), mutant template: 5'-GAT CCC TGG ACA GGC AAG GAA TAC AGG TAT TTT GT-3' (SEQ ID NO: 12). The tendencies of the two mutants to extend mismatches as compared to canonic complexes were compared. As shown in Figure 5, the QVA mutant has a substantially higher tendency to extend mismatches as compared to the LVL mutant.